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Inherent colistin resistance in Genogroups of the *Enterobacter cloacae* complex: epidemiological, genetic and biochemical analysis from the BSAC Resistance Surveillance Programme

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Running Heads: Mushtaq S *et al.* / Colistin-resistant *Enterobacter* in the UK

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Abstract.

Background. Polymyxins have re-entered use against problem Gram-negative bacteria. Resistance rates are uncertain, with estimates confounded by selective testing. **Methods**. The BSAC Resistance Surveillance Programme has routinely tested colistin since 2010; we reviewed data up to 2017 for relevant Enterobacterales (n=10,914). Unexpectedly frequent resistance was seen among the *Enterobacter cloacae* complex isolates (n=1749); for these, we investigated relationships to species, genome, carbon source utilisation and LPS structure. **Results**. Annual colistin resistance rates among *E. cloacae* complex isolates were 4.4% to 20%, with a rising trend among bloodstream organisms; in contrast, annual rates for *Escherichia coli, Klebsiella spp.* and *E. aerogenes* generally remained <2%. WGS split the *E. cloacae* complex isolates into 7 Genogroup clusters, designated A-G. Among isolates assigned to Genogroups A-D, 47/50 sequenced were colistin resistant, and many, from Genogroups A-C identified as *E. asburiae*. Isolates belonging to Genogroups E-G consistently identified as *E. cloacae* and were rarely (only 3/45 representatives sequenced) colistin resistant. Genogroups F and G – the predominant colistin-susceptible clusters – were metabolically distinct from other clusters, notably regarding utilisation or not of L-fucose, formic acid, D-serine, adonitol, *myo*-inositol, L-lyxose and polysorbates. LPS from resistant organisms grown without colistin pressure lacked substitutions with 4-amino-arabinose or ethanolamine but was more structurally complex, with more molecular species present. **Conclusions.** Colistin resistance is frequent in the *E. cloacae* complex and increasing among bloodstream isolates. It is associated with: (i) particular genomic and metabolic clusters, (ii) identification as *E. asburiae* and (iii) with more complex LPS architectures.

Introduction

After long abandonment, intravenous polymyxins have re-emerged as treatments for infections due to multidrug-resistant Gram-negative bacteria.[1,2] Colistin (polymyxin E) is also used as a nebulised agent for chronic pulmonary infections with *Pseudomonas aeruginosa*,[3] and as a non-absorbed oral agent in selective digestive decontamination.[4]

Polymyxins have multiple effects, but primarily bind to negatively-charged lipid A residues in the LPS, destabilising the outer membrane and promoting their own uptake.[5] Resistance is inherent in Proteeae, *Serratia* spp. and *Burkholderia* spp., which add positively-charged sugars (4-amino-L-arabinose, Ara4N) or amino-alcohols (ethanolamine) to their LPS, preventing polymyxin binding.[1,2,5] Resistance arises in other Enterobacterales via chromosomal mutations up-regulating systems that similarly modify the lipid A, or through acquisition of plasmids carrying *mcr* genes, encoding phosphoethanolamine transferases recruited, at least in the case of *mcr1* and *mcr2*, by horizontal transfer from *Moraxella* spp.[6,7]

The prevalence of colistin resistance in the UK and worldwide is uncertain and subject to detection bias because many laboratories only test the drug against bacteria resistant to standard agents. Moreover, mutational resistance is often unstable and readily lost, with the issue muddied by 'heteroresistance,'[8,9] the inadequacy of disc testing and the variability of MICS with the test method.[10] Despite these uncertainties it is clear and concerning that: (i) polymyxin-resistant mutants of carbapenemase-producing Enterobacterales can cause outbreaks[11] and (ii) that acquired *mcr* genes are widespread among *Escherichia coli* in food animals, e.g. in China.[12]

The BSAC Resistance Surveillance Programme[13] has tested colistin against Gram-negative bacteria (excepting inherently-resistant genera) since 2010/11. We present here the results, along with an investigation of the distribution and mode of

resistance in *Enterobacter cloacae* complex isolates, where resistance proved to be unexpectedly frequent.

Methods and materials

Isolate collection

Details of the BSAC Surveillance Programmes have been published previously.[13] Briefly, these programmes collected and tested *c.* 3000 bloodstream and lower respiratory tract infection (LRTI) isolates p.a. from centres across the UK and Ireland. The number of centres has varied from 25 to 40, with sites asked to collect fixed quotas of isolates per species group per year. There is some turnover of centres over time. Collection for the Respiratory Programme runs from October to September, to capture winter peaks in single years; the Bacteraemia Programme runs on the calendar year. Colistin began to be tested in October 2010 for LRTI isolates and January 2011 for bacteraemia isolates. Bacteraemia isolates were collected and tested by PHE's Antimicrobial Resistance and Healthcare Associated Infections Reference Unit (AMRHAI) throughout: LRTI isolates were collected and tested by Quotient BioResearch (LGC Group, Fordam, Newmarket, UK) until the 2012/13 year and thereafter by AMRHAI.

Laboratory methods

The analysis straddles a period when species identification moved from API20E® strips (bioMerieux, Basingstoke, UK) to MALDI-ToF (Bruker, Bremen, Germany); this change was introduced during the 2011-12 LRTI and 2012 Bacteraemia surveillances. In general, the direct colony method was used for MALDI-ToF, though the extraction method was employed for subsets of isolates (see Results). MALDI-ToF identification

software was updated as new versions were released - MBT DB-5627 (V.4) was employed for most testing. MICs were routinely measured by BSAC agar dilution on IsoSensitest Agar (Oxoid, Basingstoke, UK), with results interpreted *versus* 2019 EUCAST breakpoints, which score colistin as S $\leq$2 mg/L, R >2 mg/L for Enterobacterales. MICs were also determined by broth microdilution in Mueller Hinton broth for the subset of isolates where LPS structure was studied. Heteroresistance was defined as where isolated colonies 'trailed' in agar dilution or where tubes were 'skipped' in broth microdilution.

Comparison of colistin-resistant and -susceptible E. cloacae group isolates

The first 50 colistin-resistant isolates collected across both surveillances in 2010-12 were compared, in a 1:1 case : control study, with 50 randomly-selected colistin-susceptible isolates from the same centres in the same years.

WGS was undertaken using Illumina methodology, with phylogenetic analyses based on core genomes; the read length was 2x100bp and a Nextera XT DNA library Prep kit was used. Illumina pair-end sequence reads were mapped onto the genome of the type strain, *E. cloacae* ATCC13047 (GenBank accession no: NC_014121.1) using the Phoenix algorithm.[14] SNPs were called and filtered using the Genome Analysis Toolkit v2.0.[15] Maximum likelihood analyses were undertaken based on the aligned SNPs, allowing 20% of Ns and gaps, using RAxML under a GTRCAT model with 500 bootstraps.[16] The best tree was drawn using the ITOL application.[17] Sequence data supporting these analyses are in the process of being made available in the European Nucleotide Archive, under project accession number PRJEB35697.

The ability to metabolise carbon and energy sources was analysed using the BIOLOG system (Biolog, Inc., Hayward, Ca., USA) with the PM1 MicroPlate™ test

panel. Bacteria were grown overnight on CLED agar, then resuspended and loaded according to the manufacturer's directions. Results were recorded as growth curves by the OmniLog® automated incubator reader then analysed using the OmniLog® Data Collection system.

LPS analysis

Lipid A modifications were investigated using MALDI-ToF mass spectrometry (MS). Bacteria were grown overnight in LB medium without antibiotic pressure, then sedimented and washed thrice in 10 ml phosphate buffer (PB, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4). The final pellets were freeze-dried overnight then lipid A was extracted as previously described.[18] After de-salting, 2-µl lipid A aliquots were loaded onto the polished steel target of the MS instrument, air dried and covered by 1 µl of 2,5-dihydroxybenzoic acid matrix (Sigma Aldrich, Gillingham, UK) dissolved in 0.1 M aqueous citric acid, and allowed to air dry. Finally, the target was inserted in an Autoflex MALDI-ToF spectrometer (Bruker). Data acquisition and analysis were performed using the Flex Analysis software.

Statistical methods

Trends in resistance prevalence were modelled by logistic regression using robust standard errors to allow for clustering by year (e.g. due to experimental variation and batch testing) and expressed as odds ratios; P values ≤0.05 were taken as moderate evidence for trend. P≤0.05 was also considered moderate evidence for independent prediction of resistance by factors such as isolate source (blood or respiratory infection) and identity. Fisher's exact test was used to assess association between WGS group and substrate utilisation or colistin resistance, with Bonferroni adjustment for multiple

158 testing. Clustering of metabolic capabilities was investigated by k-means and compared
159 with hierarchical clustering by average linkage, using simple matching in both cases,
160 and with distinctness of clustering indicated by the Caliński-Harabasz stopping rule
161 (pseudo-F) index.
162
163 **Results**
164 *Distribution of colistin resistance in Enterobacterales*
165 Annual rates of colistin resistance among bloodstream and LRTI *Escherichia coli* and
166 *Klebsiella* species (including *K. aerogenes*) generally remained below 2% (Table 1).
167 Resistance was more prevalent in the *E. cloacae* complex, and further work
168 concentrated on this unexpected observation.
169 The prevalence of colistin resistance among bloodstream *E. cloacae* complex
170 isolates increased from 5.7% and 8.1% in 2011 and 2012, respectively, to 15.9% and
171 13.4% in 2016 and 2017, respectively; logistic regression indicated prevalence
172 increasing by a factor (odds ratio) of 1.19 per year (95% CI 1.07 to 1.32; p=0.001).
173 Resistance rates for LRTI *E. cloacae* complex isolates also were higher than for other
174 Enterobacterales species, but lacked the upward trend seen in bacteraemia (odds ratio
175 per year 0.91 with 95% CI 0.80 to 1.05; p=0.19); rather, resistance prevalence was
176 highest in the first year at 20% and thereafter fluctuated between 4.4% and 11.3%.
177 Logistic regression models did not support the source of the isolate (blood/respiratory)
178 as an independent predictor of colistin resistance after adjusting for species group.
179 MICs, by agar dilution, for the resistant isolates straddled between 4 and ≥64 mg/L,
180 compared with a sharp mode of 0.5 to 1 mg/L for susceptible isolates (Table 2).
181 Resistant isolates were collected from widely scattered sites and did not reflect
182 local outbreaks: 20/40 collecting sites submitted resistant isolates in Year 1 (i.e.

2010/11 for Respiratory and 2011 for Bacteraemia) 17/40 in Year 2, 15/40 in Year 3, 14/40 in Year 4, 17/40 in Year 5, 20/25 in Year 6 and 13/25 in Year 7.

According to their manufacturers, neither MALDI-TOF nor API20E® strips give reliable species identification within the *E. cloacae* complex. Nevertheless, and strikingly, over 85% of isolates with MICs 0.25 to 1 mg/L identified as *E. cloacae* species on first test, whereas, among isolates with higher MICs (including 2 mg/L, as the top border of susceptible), the proportion identified as *E. cloacae* was diminished: among those with MICs >64 mg/L only 24% identified as *E. cloacae* whereas 40% identified as *E. asburiae* (Table 2).

There were only minor differences among the source patients for the colistin-resistant and -susceptible Enterobacters concerning sex (58.2% male *versus* 61.8%, respectively), age distribution (≤4 years, 13.0% *versus* 10.3%, respectively; ≥60 years, 31.7% *versus* 30.0%, respectively) or ICU location (16.4% *versus* 11.5%). Resistance to several comparator antibiotics was less prevalent among the colistin-resistant than colistin-susceptible isolates, including for third-generation cephalosporins (16.4% *versus* 23.2%), ciprofloxacin (6.2% *versus* 10.3%), gentamicin (2.3% *versus* 5.7%) and piperacillin/tazobactam (11.9% *versus* 14.7%). Only a single (colistin-susceptible) isolate was resistant to carbapenems. The proportions of isolates that were non-susceptible to the various comparators was broadly stable across the surveillance period reviewed.[19]

Case : control comparison of colistin-resistant and -susceptible E. cloacae

A detailed study of the first 50 colistin-resistant Enterobacter isolates received was undertaken. These were from the 2011 and 2012 bacteraemia series and the 2010/11 and 2011/12 respiratory series and were compared with 50 colistin-susceptible controls,

208 randomly selected from among isolates collected at the same sites in the same period.
209 As in later periods, the resistant isolates came from multiple sites, including 33 of the
210 45 hospitals contributing isolates during the 2-years; 39 were from hospitals in England,
211 six from Ireland, and three each from Scotland and Wales. The maximum number of
212 resistant Enterobacters from any site was four (one site), with four further sites each
213 contributing three isolates. The susceptible controls were from 28 sites, with no more
214 than four from any one site.

215 WGS was performed on all 50 resistant isolates and 45/50 susceptible controls
216 and an MIC distribution for these is included in Table 2; sequencing failed for the
217 remaining 5 susceptible organisms. Analysis of the resulting data divided the 95
218 sequenced organisms into seven Genogroups, designated A-G, with a single outlier (fig.
219 1). Most (7/9) isolates in Genogroup A were colistin resistant, as were all those in
220 Genogroups B (n=9), C (n=16), and D (n=15). By contrast, resistance was seen in only
221 1/5 Genogroup E isolates, 2/26 Genogroup F isolates and 0/14 of Genogroup G. The *E.*
222 *cloacae* type strain NCTC10005/ ATCC13047 was colistin resistant (MIC 8 mg/L) and
223 fell into Genogroup D; the *E. asburiae* type strain NCTC12123/ATCC35953 fell into
224 Genogroup C but, exceptionally for this Genogroup, was colistin susceptible (MIC, 1
225 mg/L). None of the sequenced isolates carried acquired *mcr* genes.

226 Triplicate MALDI-ToF identification tests, using the extraction method and the
227 MBT DB-5627 (V.4) database, were performed on the 95 sequenced isolates.
228 Consistent identifications with all three replicates agreeing as *E. cloacae* dominated in
229 Genogroups D, E, F and G whereas members of Genogroups A, B and C were more
230 likely to identify repeatedly as *E. asburiae*, or to give a mixture of identifications as *E.*
231 *cloacae* and *E. asburiae* (Table 3).

Carbon source utilisation profiles were obtained for 93/100 isolates; tests for the remaining 7 isolates failed. The isolates with profiles obtained included 43 of the 45 sequenced colistin-susceptible organisms and 44/50 sequenced colistin-resistant organisms (Table 3). Further analysis, below, was based on these 87 isolates for which we had both sequence and carbon source data: it excludes 6 isolates for which we had carbon source data only and 7 for which we had sequence data only.

Strong relationships between Genogroup and metabolic profile were evident. Thus, L-fucose utilisation was seen for 36/38 Genogroup F and G isolates, also 3/5 in Genogroup E, whereas 42/43 isolates in Genogroups A-D tested could not use this sugar. The ability to utilise glucuronamide also was largely specific to Genogroup F and G, with 16/38 isolates positive *versus* 2/49 for all other Genogroups combined. Adonitol utilisation was unique to Genogroup F, though only seen for 19/25 group members; the ability to utilise lyxose also was largely specific to Genogroup F, with 12/25 isolates positive *versus* 8/62 for all other Genogroups combined. By contrast (i) the ability to utilise *myo*-inositol was near-universal across Genogroups A-E, with 46/48 isolates positive compared with 4/38 in Genogroups F and G combined, (ii) the ability to utilise formic acid was widespread in Genogroups A, B, C and E, with 34/36 isolates positive *versus* 2/51 for all other Genogroups combined, and (iii) the ability to utilise α-keto-glutaric acid was seen in over half the Genogroup A and B isolates (5/8 and 6/9, respectively) *versus* 10/70 for all other groups combined. In other cases *inability* to use a substrate was associated with particular Genogroups: thus 0/13 Genogroup G isolates utilised D-serine compared with 66/74 isolates from other Genogroups and only 2/14 Genogroup C isolates utilised D-galactonic acid-γ-lactone compared with 71/73 isolates from all other Genogroups combined.

Both the hierarchical statistical method and k-means clustering indicated that Genogroups F and G (considered as a pair) were distinct from other Genogroups in their substrate utilisation. Metabolic differences among the remaining Genogroups were not clear-cut by statistical analyses. Since Genogroups F and G also comprised the great majority of the colistin-susceptible isolates there were strong associations also between metabolic profile and susceptibility, which was strongly linked (adjusted $p < 0.01$) with the ability to utilise L-fucose, adonitol, and glucuronamide whereas the ability to utilise formic acid and *myo*-inositol was associated with resistance ($p < 0.01$).

Polysorbate (Tween®) utilisation was more widespread among colistin-resistant isolates than -susceptible isolates, with this difference more marked for polysorbate 80 (adjusted $p=0.004$) than polysorbate 40 (adjusted $p=0.011$) or polysorbate 20 (adjusted $p = 0.439$). Underlying these observations were two key traits: (i) that the great majority of Genogroup G isolates (colistin-susceptible) lacked the ability to utilise any polysorbate whereas all the predominantly colistin-resistant Genogroups (A-D) comprised organisms that mostly could utilise polysorbates 20 and 40 and, (ii) that Genogroup D (colistin resistant) and F (mostly colistin-susceptible) isolates commonly utilised polysorbates 20 and 40 but not polysorbate 80.

LPS analysis

One or two representatives of each Genogroup cluster were selected for LPS characterisation along with the *E. cloacae* type strain NCTC10005/ATCC13047, which was colistin resistant and belonged to Genogroup D. Broth dilution MICs were determined for these isolates in addition to the initial agar dilution values. The values in broth were higher, particularly for the more resistant organisms but only in one case

was there a categorical disagreement: isolate EN104606 was found susceptible on agar (and thus atypical of its Genogroup [A]) but heteroresistant in broth (Table 4).

MALDI-TOF MS analysis of the lipid A of *E. cloacae* NCTC10005/ATCC13047 (fig. 2), grown without antibiotic pressure, revealed prominent molecular ion peaks at 1387, 1797, 1840 and 2063 *m/z*. The 1387 *m/z* peak is consistent with tetra-acylated lipid A fragmentation product, while the 1824 *m/z* peak is consistent with hexa-myristoylated (C14) lipid A forms resembling those found in *K. pneumoniae*, *E. coli* and *Yersinia enterocolitica*.[20,21,22] The species at 1797 *m/z* is consistent with a penta-myristoylated lipid A substituted with an additional C12 acyl chain (fig. 2). Modified forms of the 1824 and 1797 *m/z* molecular ions were also present, reflecting addition of a hydroxyl group (typically catalysed by LpxO; *m/z* 1840 to 1824) or a palmitate group (typically catalysed by PagP) (*m/z* 2063 to 1824).[24] These results indicate that the lipid A of strain ATCC13407 is complex and modified by 2-hydroxylation and palmitoylation. No molecular ions consistent with Ara4N or phosphoethanolamine substitutions were observed using bacteria grown in drug-free LB; an analysis of lipid A modifications under other *in vitro* growth conditions and *in vivo* will be reported elsewhere.

Lipid A analyses for representatives of the various DNA Genogroups are summarised in Table 4. The 1824 *m/z* molecular ion was abundant in the lipid A of most isolates except N2878 (Genogroup E) (Table 4). Most of the isolates produced penta-myristoylated lipid A with an additional C12 acyl chain (1797 *m/z*), although the abundance of this ion varied (Table 4 and fig. S1). In general, more complex patterns, with a greater number of molecular species, particularly including modified forms with hydroxylation and palmitoylation, were observed for isolates with colistin resistance or heteroresistance (Table 4).

Discussion

Colistin began to be tested in the BSAC Surveillance in 2010 and we reviewed subsequent resistance trends among Enterobacterales. Resistance prevalence remained under 2% in almost all years for *E. coli* and *Klebsiella* (including *K. aerogenes*) but was higher for bloodstream and respiratory *E. cloacae* complex isolates, with a strong increasing trend for bloodstream, but not respiratory, organisms. The colistin-resistant *E. cloacae* isolates were widely scattered in time and place and did not represent outbreak clones, as confirmed by WGS. Rather, they largely belonged to Genogroups A-D and had metabolic differences from members of Genogroups F and G, which, along with Genogroup E, encompassed the great majority of colistin-susceptible *E. cloacae*. Within Genogroups A-D, 47/49 isolates were colistin resistant compared with 3/45 isolates in Genogroups E-G.

Relating our Genogroups to the established taxonomy for *the E. cloacae* complex proved challenging. Although MALDI-ToF does not reliably identify within the *E. cloacae* complex it was striking that isolates belonging to three of the four predominantly colistin-resistant Genogroups (A, B and C but not D) were more likely to identify as *E. asburiae*, or to give a mixture of *E. asburiae* and *E. cloacae* identifications, with this likelihood rising with the colistin MIC. Conversely, isolates belonging to the predominantly susceptible Genogroups E, F and G largely identified as *E. cloacae*, as did the (mostly resistant) members of Genogroup D. The simple notion of intrinsic colistin resistance in *E. asburiae* and susceptibility in *E. cloacae* is however refuted by the literature[25] and by the type strains *E. asburiae* NCTC12123/ATCC35953 (Group C, MIC 1 mg/L) and *E. cloacae* NCTC10005/ATCC13047 (Group D, MIC 8 mg/L) showing the converse pattern.

Grimont and Grimont write, in *Bergey's Manual of Determinative Bacteriology*[26] that 'typical' *E. cloacae* largely fall into their genomic group 3. This putatively corresponds to our Genogroups F and G, as evidenced (i) by the dominance of these genomic groups among 'typical' colistin-susceptible isolates and (ii) by ability of Genogroup F and G isolates to utilise adonitol and fucose, which are not utilised by Bergey's other genomic groups - and inability to utilise *myo*-inositol- a substrate Bergey notes as less reliably used by group 3 than by other groups. Our Genogroups A-D predominantly comprised organisms selected on the basis of their colistin resistance – a trait unlikely to be common in series selected on other criteria –so it is perhaps unsurprising that they do not obviously match to other genomic groups described by Grimont and Grimont. We agree that the 'type strain' *E. cloacae* NCTC10005/ATCC13047 is poorly representative, as it clusters apart from typical (i.e. F and G / group 3) isolates, falling into our Genogroup D and Grimont and Grimont's group 1.

In general, colistin resistance depends on the production of modified lipid A molecules with substitutions that reduce the electronegative potential of the LPS. The lipid A profiles of selected isolates representing the various *E. cloacae* clusters were investigated after growth without antibiotic pressure to assess if isolates displaying high resistance or heteroresistance to colistin were "primed" for resistance. Compared with susceptible isolates, the resistant and heteroresistant organisms examined exhibited complex lipid A patterns, more often having forms with 2-hydroxylation and palmitoylation, which typically are generated by LpxO and PagP enzymes. These modifications are associated, in several bacteria, with enhanced resistance to polymyxins and vertebrate antimicrobial peptides,[27-29] and they are predicted to reduce permeability across the outer membrane.[29] The expression of *lpxO* and *pagP* genes is

355 regulated by the master two-component system PhoPQ,[30,31] which controls enzymes
356 involved in the remodelling of the LPS, including those that add Ara4N and
357 ethanolamine. Although expression of *lpxO* and *pagP* homologues was not investigated
358 here, our results suggest that the heteroresistant isolates have a higher basal level of
359 expression of these genes, which might confer some immediate protection against
360 colistin, with this further enhanced by full activation of the PhoPQ regulon in the
361 presence of the antibiotic. Recent work suggests that LpxO expression is also regulated
362 by the metabolic and redox status of the bacterium,[32] suggesting that heteroresistance
363 could also reflect bacterial adaptation to metabolic stress.

364 The study has several limitations, besides the taxonomic uncertainty within the
365 *E. cloacae* complex. First, agar dilution was used for susceptibility testing whereas
366 broth microdilution is now recommended by EUCAST as more reliable for resistance
367 detection, though this view is disputed by others.[10] At most, however, this may have
368 led us to underestimate the prevalence and degree of resistance; for the isolates
369 subjected to LPS analysis (Table 4) broth MICs were determined, in addition to the
370 original agar values. The broth values typically were higher but, only in one case did
371 this lead to a categorical disagreement: isolate EN104606 was found susceptible – and
372 so atypical of Genogroup A, to which it belonged, by agar dilution – but proved
373 heteroresistant in broth testing. A second caveat is that colistin MICs were very widely
374 distributed among resistant isolates and an organism with an MIC of 4 mg/L may have
375 more in common with a 'susceptible' *E. cloacae* than one with an MIC of >64 mg/L.
376 Third, this project had a long gestation and WGS and metabolic profiling were
377 exclusively done on isolates from the early years. It is plausible that proportions of the
378 different Genogroups changed subsequently and that expansion of one or more of this

predominantly resistant groups A-D, with particular pathogenic traits, explains the dichotomy in trends for bloodstream and LRTI isolates.

The practical importance of our observations are uncertain. On the one hand, *E. cloacae* is among the more frequent Gram-negative opportunists and, along with *E. coli* and *K. pneumoniae*, is among the major hosts of carbapenemases, forcing colistin use. Accordingly, any potential 'loss' of colistin, e.g. via an expansion of one or more resistant Genogroup, would potentially be concerning. On the other hand, intravenous polymyxins are now being supplanted by β-lactamase inhibitor combinations, including ceftazidime/avibactam and meropenem/vaborbactam, which are less toxic and appear more efficacious against Enterobacterales with KPC (and OXA-48 in the case of ceftazidime/avibactam) carbapenemases.[33] If these and coming analogues including imipenem/relebactam, cefiderocol and cefepime/zidebactam live up to their promise, the renaissance of intravenous polymyxins may prove to be a brief interlude, and the erosion of *Enterobacter* coverage less than catastrophic.

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Transparency declarations:

DML: Advisory Boards or ad-hoc consultancy Accelerate, Allecra, Antabio, Centauri, Entasis, Integra-Holdings, Meiji, Melinta, Menarini, Mutabilis, Nordic, ParaPharm, Pfizer, QPEX, Roche, Shionogi, T.A.Z., Tetraphase, VenatoRx, Wockhardt, Zambon, Paid lectures – Astellas, bioMerieux, Beckman Coulter, Cardiome, Cepheid, Merck/MSD, Menarini, Nordic, Pfizer and Shionogi. Relevant shareholdings or options – Dechra, GSK, Merck, Perkin Elmer, Pfizer, T.A.Z, amounting to <10% of portfolio value. **SM, RA, AC, BP:** nothing to declare but PHE's AMRHAI Reference Unit has received financial support for conference attendance, lectures, research projects or contracted evaluations from numerous sources, including: Accelerate, Achaogen, Allecra, Amplex, AstraZeneca, AusDiagnostics, Basilea, Becton Dickinson, bioMérieux, Bio-Rad Laboratories, BSAC, Cepheid, Check-Points B.V., Cubist, Department of Health, Enigma Diagnostics, ECDC, Food Standards Agency, GenePOC™, GlaxoSmithKline, Helperby Therapeutics, Henry Stewart Talks, IHMA, Innovate UK, Kalidex Pharmaceuticals, Melinta Therapeutics, Merck Sharpe & Dohme, Meiji Seika, Mobidiag, Momentum Biosciences, Neem Biotech, NIHR, Nordic Pharma, Norgine Pharmaceuticals, Rempex Pharmaceuticals, Roche, Rokitan, Smith & Nephew, Shionogi, VenatoRx Pharmaceuticals, Wockhardt Ltd and the WHO. **OE**, conference support from Pfizer; **JM** shareholdings and options – Thermo Fisher

Scientific, amounting to <10% of portfolio value; **MCG, IGR, TLB, MAV, RR, MD, CH:** nothing to declare.

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528 **Table 1.** Rates of resistance to colistin among Enterobacterales species collected in the BSAC Surveillance Programme
529

	Proportion of isolates found resistant to colistin, 2 mg/L						
	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7
Bacteraemia	2011	2012	2013	2014	2015	2016	2017
*Enterobacter cloacae group**	**9/158**	**13/161**	**12/160**	**11/172**	**17/169**	**27/170**	**21/157**
K. aerogenes (E. aerogenes)	0/14	0/36	0/44	0/36	1/41	1/29	1/29
K. pneumoniae	2/200	4/196	1/213	1/205	**8/206**	2/186	3/164
K. oxytoca	0/56	0/55	0/53	0/55	0/57	0/58	0/57
E. coli	2/522	3/520	6/539	3/547	3/548	1/496	2/477
LRTI	2010/11	2011/12	2012/13	2013/14	2014/15	2015/16	2016/17
*Enterobacter cloacae group**	**21/105**	**7/93**	**10/85**	**3/68**	**9/90**	**10/90**	**8/71**
K. aerogenes (E. aerogenes)	0/34	0/42	0/40	0/22	0/23	0/35	0/32
K. pneumoniae	**4/145**	**4/166**	2/140	3/150	**4/187**	0/152	2/141
K. oxytoca	**3/71**	1/73	1/56	0/68	0/63	1/68	0/49
E. coli	**6/274**	1/239	0/250	0/255	0/244	0/230	3/277

530 Bold text indicates rates >2%
531 **Enterobacter cloacae* group comprises: *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*,
532 *Enterobacter ludwigii* and *Enterobacter nimipressuralis*.
533 Until 2014/15 (respiratory) and 2015 (bacteraemia) isolates were collected from up to 40 centres across the UK and Ireland and, thereafter from
534 up to 25 centres.
535

536 **Table 2.** Species identification within the *E. cloacae* group in relation to colistin MIC: all isolates.
537

Colistin MIC (mg/L)	Whole collection 2010-17: (%) isolates identified as:					Isolates sequenced
	Total	*E. cloacae*	*E. asburiae*	Other named	No species-level identification	
≤0.25 (S)	184 (100)	169 (91.8)	8 (4.3)	6 (3.3)	1 (0.5)	21
0.5 (S)	990 (100)	880 (88.9)	54 (5.5)	3 (0.3)	53 (5.4)	23
1 (S)	360 (100)	311 (86.4)	20 (5.6)	2 (0.6)	27 (7.5)	1
2 (S, breakpoint))	37 (100)	24 (64.9)	9 (24.3)	0 (0)	4 (10.8)	
4 (R)	26 (100)	13 (50.0)	10 (38.5)	1 (3.8)	2 (7.7)	7
8 (R)	31 (100)	21 (67.7)	6 (19.4)	0 (0)	4 (12.9)	8
16 (R)	25 (100)	17 (68.0)	2 (8.0)	3 (12.0)	3 (12.0)	12
32 (R)	21 (100)	10 (47.6)	2 (9.5)	1 (4.8)	8 (38.1)	
≥64 (R)	75 (100)	18 (24.0)	30 (40.0)	2 (2.7)	25 (33.3)	23
Total	1749 (100)	1463 (83.6)	141 (8.1)	18 (1.0)	127 (7.3)	95[a]

538

539 [a] Omits 5 isolates for which sequencing failed

Note: From 2010 to 2012 identification was by API20E strips; subsequently identification was by MALDI-ToF using the colony method (see text). Neither method gives reliable definitive identifications within the *E. cloacae* complex and 'identifications' were apt to vary upon repeat testing; nevertheless the trend to higher proportion identified as *E. asburiae* or not identified to species level at higher colistin MIC is clear.

544 **Table 3**. Metabolic traits in relation to colistin status and WGS-based Genogroup

	In relation to Colistin MIC (mg/L)		In relation to Genogroup							
	≤2	>2	A	B	C	D	E	F	G	Outlier
No. group	43	44	8	9	14	12	5	25	13	1
No. able to metabolise										
Dulcitol	3	8	1	5	1	1	0	2	0	1
D-Serine	27	39	8	5	13	12	5	22	0	1
L-Fucose	37	3	0	0	0	1	3	23	13	0
Formic acid	6	30	8	9	12	2	5	0	0	0
D-Galactonic acid-γ-lactone	42	31	8	8	2	12	5	25	13	0
L-Rhamnose	43	38	8	9	8	12	5	25	13	1
D-Melibiose	36	42	7	9	13	12	5	24	8	1
α-Keto-glutaric acid	6	15	5	6	3	0	0	6	1	0
α-Methyl-D-galactoside	37	42	7	9	13	12	5	24	8	1
Lactulose	20	13	6	1	5	2	2	9	8	0
α-Hydroxy butyric acid	9	4	1	0	2	1	1	5	3	0
Adonitol	17	2	0	0	0	0	0	19	0	0

Glycyl-L-aspartic acid	40	36	8	9	13	6	3	25	12	5
myo-Inositol	9	42	7	9	13	12	5	4	0	1
Mono-methyl succinic acid	4	10	3	0	6	1	0	3	1	0
L-Lyxose	16	4	0	2	1	0	1	12	3	1
Glucuronamide	16	2	1	0	1	0	0	9	7	0
Phenylethylamine	5	2	0	1	0	0	0	5	1	0
Polysorbate (Tween®) 20	23	35	7	9	11	8	5	16	2	0
Polysorbate (Tween®) 40	20	37	7	9	11	10	5	12	3	0
Polysorbate (Tween®) 80	10	28	6	8	10	4	5	4	1	0
Identifications: based on top scores by 3 tests by MALDI-ToF										
E. cloacae in 3/3 cases	36	15	2	0	0	10	4	23	12	0
E. asburiae in 3/3 cases	0	17	2	4	10	1	0	0	0	0
Mixed results	7	12	4	5	4	1	1	2	1	1

Substrates tested and metabolised by >90% of both colistin-susceptible and –resistant isolates: acetic acid, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, adenosine, D-alanine, L-alanine, L-alanyl-glycine, L-arabinose, L-asparagine, L-aspartic acid, bromosuccinic acid, D-cellobiose, citric acid, 2-deoxy adenosine, D-fructose, D-fructose-6-phosphate, fumaric acid, L-galactonic acid-γ-lactone D-galacturonic acid, D-galactose, D-gluconic acid, α-D-glucose, D-glucose-1-phosphate, D-glucose-6-phosphate, D-glucuronic acid, L-glutamic acid, L-glutamine, glycerol, D,L–α-glycerol phosphate, glycyl-L-glutamic acid, glycyl-L-proline, *m*-hydroxy-phenylacetic acid, *p*-hydroxy-phenylacetic acid, inosine, L-lactic acid,

α-D-lactose, D,L-malic acid, L-malic acid, maltose, maltotriose, D-mannitol, D-mannose, β-methyl-D-glucoside, methyl pyruvate, mucic acid, L-proline, pyruvic acid, D-ribose, D-saccharic acid, L-serine, D-sorbitol, succinic acid, sucrose, thymidine, L-threonine, D-trehalose, uridine and D-xylose. Compounds metabolised by <10% of both colistin-susceptible and –resistant isolates were acetoacetic acid, 2-aminoethanol, D-aspartic acid, D-glucosaminic acid, glycolic acid, glyoxalic acid, α-hydroxy glutaric acid-γ-lactone, α–keto-butyric acid, D-malic acid, 1,2 propanediol, propionic acid, D-psicose, m-tartaric acid, D-threonine, tricarballylic acid and tyramine.

Table 4. Main lipid A species present in representative isolates in relation to colistin MIC and Genogroup

Isolate	Genogroup	Colistin MIC (mg/L)		*m/z* ion peaks observed by MALDI-TOF mass spectrometry[b]					
		Agar (BSAC)	Broth (Belfast)	1387	1797	1824	1840	2036	2063
EN104606	A	0.5	4 to 32[a]	+	+	+	+	+	+
EN2852	A	256	512	+	+	+	+	+	+
EN104107	B	4	512	+	+	+	-	?[c]	?[c]
EN105227	B	8	>1024	+	+	+	+	+	+
EN100708	C	>32	256	+	+	+	-	-	-
EN105406	C	>32	32 to 1024[a]	+	+	+	+	+	+
EN2692	D	16	512	+	+	+	+	+	+
NCTC/10005 ATCC13047	D	8	128	+	+	+	+	+	+
EN2720	D	4	8 to 512[a]	+	+	+	+	+	+
EN2878	E	0.5	2	-	+	-	-	+	-
EN2889	F	0.5	2	+	+	+	+	-	+
EN104619	F	0.25	2	+	+	+	+	-	+
EN104003	F	8	4	-	-	+	-	-	-
EN115203	G	0.25	2	+	+	+	-	-	-

[a] Substantial heteroresistance / trailing end-points seen

[b] Proposed lipid A composition of each of the molecular ions (see fig. S1 for detailed spectra):

1387; Tetra-acyl (3x C14:0(3-OH), 1x C14:0), 2P

1797; Hexa-acyl (4x C14:0(3-OH), 1x C14:0, 1x C12:0), 2P

1824; Hexa-acyl (4x C14:0(3-OH), 2x C14:0), 2P

1840; Hexa-acyl (4x C14:0(3-OH), 1x C14:0, 1x C14:0(3-OH), 2P

2036; Hepta-acyl (4x C14:0(3-OH), 1x C14:0, 1x C12:0, 1x C16:0), 2P

2063; Hepta-acyl (4x C14:0(3-OH), 2x C14:0, 1x C16:0), 2P

[c] Molecular ion could not be confirmed due to background noise

572 **Figure Legends**

573 **Figure 1.** Dendrogram showing relatedness of the 95 *E. cloacae* group isolates
574 successfully sequenced together with type strains *E. cloacae*
575 NCTC10005/ATCC13047 (Genogroup D) and *E. asburiae* NCTC12123/ATCC35953
576 (Genogroup C).

577

578 Footnotes

579 A-G, Genogroups described in the text.

580 Open circle, colistin-susceptible isolate; solid circle, colistin-resistant isolate

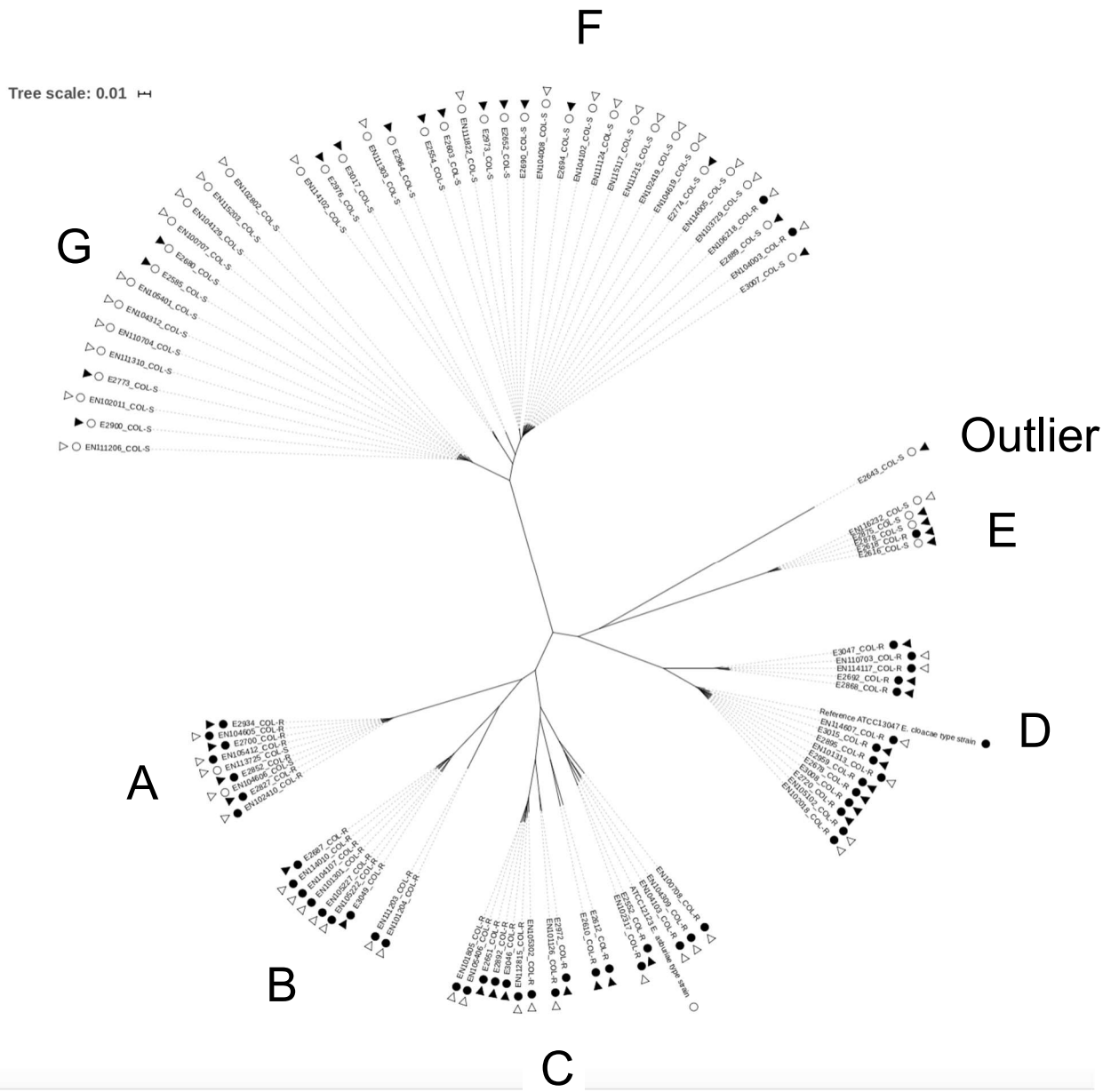
581 Open triangle, respiratory isolate; solid triangle, bloodstream isolate

582

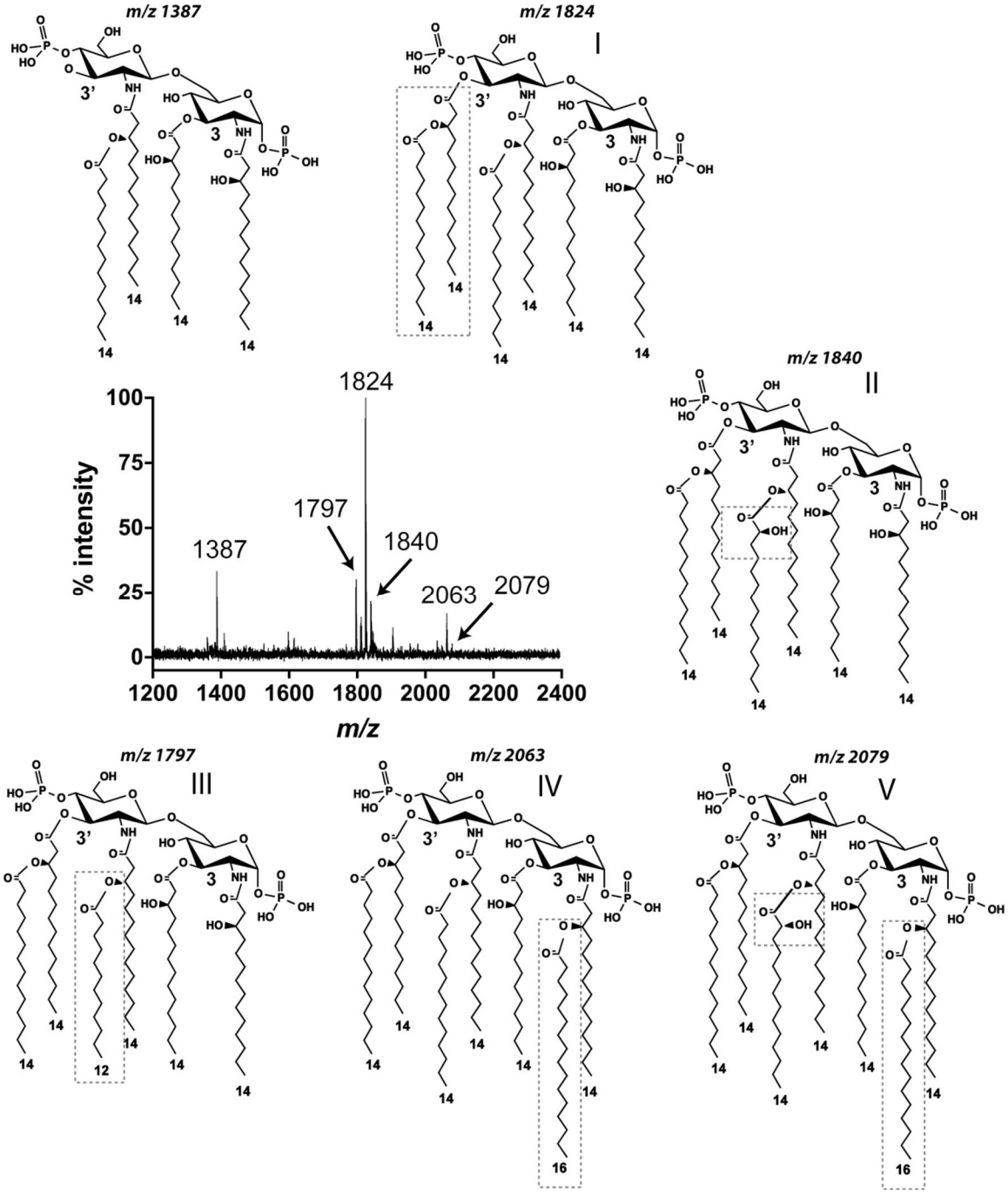
583 **Figure 2.** MALDI-TOF mass spectrometry and predicted lipid A species in the *E.*
584 *cloacae* ATCC13047 type strain. The prominent molecular ion in the spectrum (*m/z*
585 1824) corresponds to a di-phosphorylated hexa-acylated lipid A, which is identical to
586 that described for *Klebsiella pneumoniae*.[22] Dotted squares indicate the following
587 modifications likely responsible for the observed mass shifts: I, elimination of the
588 myristoxymyristoyl group (*m/z* 1387) by fragmentation of *m/z* 1824);[23] II,
589 hydroxylation of the C'-2 myristoyl-oxo-acyl chain (*m/z* 1840);[22] III, C'-2 lauryl-oxo-
590 acyl chain (*m/z* 1797); IV, palmitoylation of the C-1 acyl-oxo-acyl chain (*m/z* 2063);
591 V, hydroxylation of the C'-2 myristoyl-oxo-acyl chain plus palmitoylation of the C-1
592 acyl-oxo-acyl chain (*m/z* 2079).

593

Figure 1


Tree scale: 0.01
F
G
Outlier
E
D
A
B
C
Reference ATCC13047 E. cloacae type strain
ATCC12121 E. asburiae type strain

597 **Figure 2.**



598